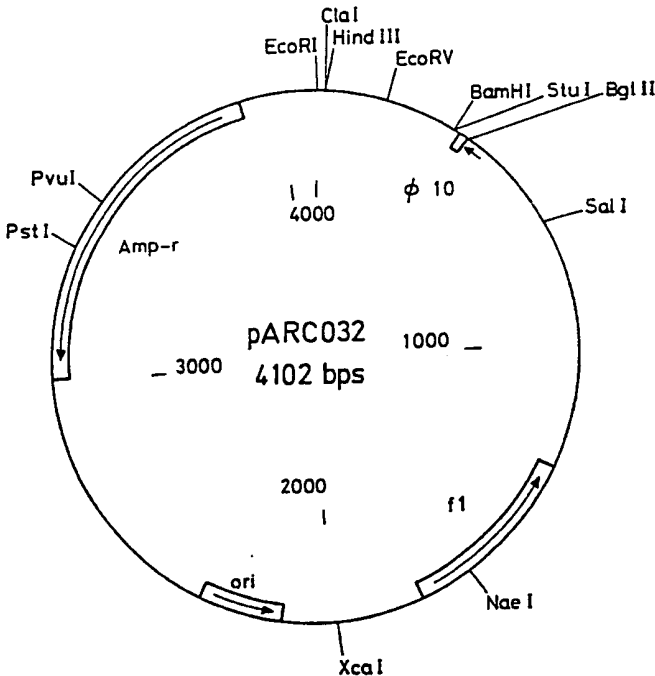


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<p>(21) International Application Number: PCT/SE92/00793 (22) International Filing Date: 19 November 1992 (19.11.92) (30) Priority data: 9124556.3 20 November 1991 (20.11.91) GB (71) Applicant (for all designated States except US): AB ASTRA [SE/SE]; S-151 85 Södertälje (SE). (72) Inventors; and (75) Inventors/Applicants (for US only) : DWARAKANATH, Prahallad [IN/IN]; 82, 6th Main, 18th Cross Road, Mal- leswaram, Bangalore-560055 (IN). DAS, Goutam [IN/ IN]; Shringar Apt. No. 4, 18th Cross Road, Malleswa- ram, Bangalore-560055 (IN).</p>		<p>(74) Agents: DANIELSSON, Sten et al.; AB Astra, Patent De- partment, S-151 85 Södertälje (SE). (81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, UA, US, Eu- ropean patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG). Published With international search report.</p>
<p>(54) Title: PHASMID VECTOR IN <i>E. COLI</i></p> <p>(57) Abstract</p> <p>The construction of a novel phasmid vec- tor which can be used as a general cloning vehi- cle is described. Upon transformation of <i>E. Coli</i> male specific strains with this novel phasmid containing a recombinant insert, ss phasmid DNA packaged as a viral particle can be pro- duced when the transformants are superinfected with a helper phage. This ss phasmid DNA could also be used as template for ss DNA sequencing and <i>in vitro</i> site directed mutagenesis. At appro- priate condition of insertion of the gene and by proper choice of host strain, this novel phasmid has the capability to hyperexpress the inserted gene.</p> 		

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PHASMID VECTOR IN *E.COLI*

TECHNICAL FIELD

5

Disclosed is the construction of a novel phasmid vector of *E.coli* which can be used for cloning gene(s) of interest and for hyperexpression of gene products in suitable host bacteria. This vector can, when incorporated into a "male specific" *E.coli* strain and upon superinfection with helper phage, give rise to single stranded plasmid DNA which can be sequenced directly by dideoxy sequencing methods and which also can be used as a template for site directed *in vitro* mutagenesis.

10

15 BACKGROUND ART

The manufacture of a large number of recombinant DNA molecules have been due to the concurrent development in the construction of vectors for cloning and manipulation of DNA. These vectors are generally characterised by the presence of a genetic determinant, the phenotype of which is altered by insertional inactivation. In addition, these vectors contain multiple restriction enzyme cleavage sites allowing the direct cloning of a variety of restriction fragments.

20

The first cloning vector constructed was pBR322 (A survey of Molecular Cloning Vectors and their uses, 1988, Biotechnology Series, Boskin, Butterworth, Rodrigues, M. & Denhardt D. (eds)). This vector could be used only for cloning purposes and not for manipulating the expression of inserted gene. For such purposes, a number of inducible expression systems were developed. An example of such an expression system is the T7 system, developed by Studier & Moffat (1986, J.Mol.Biol. 189, 113-). This system makes use of the high specificity of the T7 RNA

25

30

polymerase to recognise and transcribe any gene downstream of the T7
Ø 10 promoter.

5 When studying gene structure and function, techniques such as DNA
sequencing and site directed *in vitro* mutagenesis are efficiently carried
out on single stranded (ss) DNA templates. In 1981, Dotto *et al.*,
(Virology 114, 463-) showed that a plasmid carrying the intergenic region
(IG) of filamentous phage f1 could be packaged as ss DNA into a viral
10 particle by a helper phage. On the basis of these observations, a number
of cloning vectors have been developed which contain the IG region of a
filamentous phage, such as pEMBL (Dente *et al.*, 1983, Nucl. Acid Res.
11, 1645-), Blue scribe (Stratagene, San Diego, CA, USA), pGEM
(Promega, Madison, WI, USA), *etc.*

15 Utilizing the same logic, a second generation of pUC vectors such as
pUC118 and pUC119 which can give rise to ss plasmid DNA upon
superinfection with helper phage have also been developed (Viera &
Messing, 1987, Methods Enzymol. 153, 3-). In a similar fashion, a series
of cloning vectors with the property of releasing ss plasmid upon
20 superinfection with helper phage have been developed by Konings *et al.*
(pKUN series) where the specific DNA sequences of the filamentous
phage Ff and the like are utilized (Konings *et al.*, 1987, Methods
enzymol. 153, 12-; Peeters, B.P.H., Konings, R.N.H. & Schoenmakers,
J.G.G. 1987). All these plasmid vectors, which have the capability of
25 producing ss plasmid DNA packaged inside a viral coat upon
superinfection with another helper phage, are commonly referred to as
phasmid vectors.

30 The vectors mentioned above are, however, suitable only for general
cloning purposes. Phasmid vectors which can be utilized also for
expression are very few at present. One of them, pFSE4, employs a λ -
phage promoter (P_L) including the cII ribosome binding site (RBS).

Under appropriate conditions, insertion can hypothetically be expressed in this vector, although this vector is not considered as a hyperexpression vector (Cloning vectors. A laboratory manual, Pouwels *et al.* (eds.), 1986). The other phasmid expression vector belongs to the pSP6 family, where the promoter of a *Salmonella* phage gene is utilized for the expression of the cloned gene (Cloning vectors. A laboratory manual, Pouwels *et al.* (eds), 1986).

10 PURPOSE OF INVENTION

The purpose of the invention is to incorporate the above mentioned individual features of known vectors into a single vector. The invention comprises a phasmid vector which contains the phage f1 origin of replication and which can be used for general cloning, production of single stranded plasmid DNA and for hyperexpression of the cloned gene under appropriate host condition. The phasmid vector according to the invention is a genetic engineering tool, which is used for cloning, sequencing and hyperexpression of a gene.

20

DISCLOSURE OF INVENTION

In order to develop a phasmid vector with the ability to hyperexpress any cloned gene and the ability to produce ss DNA of the said gene, the following features were included in the plasmid:

- a. Introduction of the origin of replication of the single stranded DNA phage f1 which will enable the ss plasmid DNA to be packaged into the viral particle.
- 30 b. Introduction of a strong promoter preceding the cloning site(s) for expression of the cloned gene.

- c. Introduction of an antibiotic resistance marker for proper selection and maintenance of the plasmid in *E.coli* host.
- d. Introduction of an *E.coli* origin of replication for propagation of the plasmid in *E.coli*.

5

To achieve the desired vector construction, *E.coli* pET7 plasmid (Fig. 1; Studier and Moffat, 1986, J.Mol.Biol. 189, 113-) was used as a source of the T7 Ø 10 promoter of Bacteriophage T7 which is specifically recognized by T7 RNA polymerase. For the construction of the plasmid according to the invention, pET7 was digested with *PvuII* and *NruI* and the large fragment was purified. In the next step, the origin of the replication of *E.coli* f1 was introduced into this plasmid. The fragment containing the f1 origin of replication was excised out of pGEM7Zf(+) (Promega Corporation Catalogue; Yanish-Perron *et al.* (1985) Gene 33, 103-109) plasmid by digestion using the restriction enzyme *Sau3AI*. Subsequently the restriction fragments were filled-in using the Klenow DNA polymerase. A 788 bp fragment separated on agarose gel contained the f1 origin of replication. This fragment was ligated to the *PvuII* and *NruI* digested large fragment of pET7 to generate pARC032 and pARC035 (Figs. 2 and 3).

20

SPECIAL FEATURES OF THE PHASMID VECTOR

- 25 1. pARC032 and pARC035 in *E.coli* male specific hosts upon superinfection with helper phage R408 (Russel *et al.*, 1986, Gene 45, 333) can package alternative strands of the plasmids as ss DNA into the virus particle.
2. Both the plasmids pARC032 and pARC035 can be used as cloning vectors.

30

3. The gene of interest can be cloned into any of the unique multicloning sites (*e.g.* *Bam*HI, *Eco*RV, *Hind*III, *Cl*al and *Eco*RI) available following the T7 Ø 10 promotor element.
4. The cloned gene can be overexpressed in the presence of a functional T7 RNA polymerase using suitable host systems.
5. Upon introduction of these plasmids into *E.coli* JM101, the transformed strains can be superinfected with the helper phage and ss DNA can be isolated for sequencing purposes and/or for other manipulations.
6. Plasmid vector with Kanamycin marker (*eg.* pARC036) can be used to derive ss plasmid DNA which can be used as a template for *in vitro* mutagenesis.

EXAMPLE 1 — Construction of pARC036

In order to be able to utilize the ss plasmid DNA, which can be generated from the plasmids according to the invention, as a template for *in vitro* mutagenesis, the Ampicillin resistance marker of pARC032 was replaced with the Kanamycin resistance cartridge, since the *E.coli* CJ236 used in the preparation of the template for *in vitro* mutagenesis sometimes become spontaneously ampicillin resistant. A plasmid containing the kanamycin resistance gene was digested with *Pst*I and the 1.3 Kb fragment containing the Kan^R gene was purified. The fragment was blunt ended with bacteriophage T4 DNA polymerase. This fragment was ligated to the large fragment of *Eco*RI and *Pst*I digested pARC032 following T4 DNA polymerase treatment. The ligated product was used to transform *E.coli* HB101 and kanamycin resistant colonies were selected and the plasmid was characterized. This plasmid pARC036 (Fig. 4) has the T7 Ø 10 promoter, f1 origin of replication, as well as the Kan^r marker. The construction of pARC032 and pARC035 is shown in Fig. 5 and that of pARC036 is shown in Fig. 6.

EXAMPLE 2 — Cloning of *E.coli st* gene in pARC036

5 The utility of the plasmid pARC036 was checked by inserting an *E.coli* plasmid gene at the *Bam*HI - *Hind*III site of pARC036. The gene cloned was *E.coli st* gene as a *Bam*HI - *Hind*III fragment (Dwarakanath *et al.*, 1989, Gene 81, 219-). The cloning strategy is shown in Fig. 7 and the final construct is shown in Fig. 8. Advantage of this cloning strategy was that if the *Hind*III sites of the vector pARC036 were used, upon
10 successful cloning in a 3-way ligation process the Kan^r property should be restored. The resulting plasmid containing the *st* gene insert is designated as pARC044 (Fig. 8). The plasmid pARC044 was used to transform *E.coli* JM101, BL21 (DE3) and CJ236. The transformed strains maintained the plasmid stably. Upon superinfection with helper phage
15 R408, the JM101 (pARC044) produced ss plasmid DNA containing the *st* gene insert which can be sequenced following the isolation of the ss plasmid DNA according to the known standard procedure (Molecular Cloning, Maniatis *et al.* (eds) CSH Publ. 1989).

20

EXAMPLE 3 — Hyperexpression of *st* gene cloned in the novel phasmid vector

E.coli hyperexpression strain BL21 (DE3) which has the T7 RNA
25 polymerase gene cloned under the lac UV5 promoter at the chromosomal locus (Studier and Moffat, 1986, J.Mol.Biol. 189, 113-) was transformed with pARC 044. The transformed clones were characterized and further grown in M9 media (Dwarakanath *et al.*, 1989, Gene 81, 219-) and were induced to hyperexpress ST as a secreted product according to the
30 procedure previously described by Dwarakanath *et al.* (1989, Gene 81, 219-). The level of secreted ST under induced condition was comparable

to the wild-type level as determined by a previously standardized ELISA method (Dwarakanath *et al.*, 1989, Gene 81, 219-).

5 **EXAMPLE 4 — ss phasmid DNA as a template for site directed *in vitro* mutagenesis**

E.coli CJ236 (dut, ung) was transformed with pARC044 and the transformants were characterized. One of the transformants was grown
10 in liquid medium and was superinfected with helper phage. After overnight incubation at 37°C with vigorous shaking, the culture supernatant containing the phasmid, virions were separated from the bacterial pellet, and the ss plasmid DNA was isolated according to Maniatis. (Molecular Cloning, Maniatis *et al.*, (eds) CSH Publ., 1989). As
15 shown in Fig. 9, this phasmid DNA was used as a template to introduce a mutation in the *st* gene.

20 **THE INVENTION CAN BE SUMMARIZED IN THE FOLLOWING CLAUSES:**

1. Vector phasmids pARC032, pARC035 constructed from a starting vector incorporating
25 (a) in opposite orientation, relative to the T7 Ø 10 promoter, the replication origin and a morphogenetic signal, or an active mutant thereof, of a filamentous bacteriophage of *E.coli* bacteria having F pili; and
 (b) T7 phage promoter followed by a multiple cloning site.
2. Vector phasmids pARC032, pARC035 which contain the replication
30 origin and a morphogenetic signal, or an active mutant thereof, of the filamentous bacteriophage Ff (M13, fd and f1) or a mutant of said bacteriophage.

3. Vector phasmids pARC032 and pARC035 in which the ampicillin resistance phenotype has been replaced by kanamycin resistance phenotype as exemplified by the construction of pARC036 from pARC035.
- 5 4. Vector phasmid pARC 032 having the structure shown in Figure 2.
5. Vector phasmid pARC 035 having the structure shown in Figure 3.
6. Vector phasmid pARC032 and pARC035 into which a fragment of double-stranded DNA has been inserted at the multiple cloning site.
- 10 7. Vector phasmids pARC032 and pARC035 which can replicate in a bacterial host.
8. Vector phasmids pARC032 and pARC035 which can produce single stranded DNA of a gene inserted at the multiple cloning site in an appropriate host cell upon superinfection by a helper phage.
- 15 9. Vector phasmids pARC032 and pARC035 wherein a synthetic oligonucleotide containing a mismatch flanked by complementary sequences to the gene is inserted at the multiple cloning site.
10. Vector phasmids pARC032 and pARC035 capable of producing ss DNA under standard culture conditions which can be labelled with radioactive elements such as ^{32}P or other chemical markers suitable for determining the nucleotide sequence of the ss DNA.
- 20 11. Vector phasmids pARC032 and pARC035 as in paragraph 1 which upon insertion in a suitable host cell, can be selected by growing the host cell in kanamycin containing medium.
- 25 12. Vector phasmids pARC032 and pARC035 wherein the new restriction sites could be introduced into the multiple cloning site by standard method of *in vitro* site directed mutagenesis.
13. Vector phasmids pARC032 and pARC035 which contains a bacteriophage T7 promoter for transcription and which is transcribed *in vivo* or *in vitro* by a T7 RNA polymerase.
- 30

14. A bacterial cell, transformed by the vector phasmids pARC032 or pARC035, which produces a protein or a peptide encoded by a gene inserted at the multiple cloning site.
- 5 15. A bacterial cell, transformed by the vector phasmids pARC032 or pARC035, which produces a mutagenized protein or peptide encoded by the mutagenized ss DNA.
16. A process for the production of a protein or a peptide, encoded by the gene inserted at the multiple cloning site, by using a bacterial cell transformed by the vector phasmids pARC032 or pARC035.
- 10 17. A process for the production of a mutagenized protein or peptide, encoded by the mutagenized ss DNA, by using a bacterial cell transformed by the vector phasmids pARC032 or pARC035.

15 Further aspects of the invention can be summarized in the following clauses:

1. A phasmid construct containing a DNA phage f1 origin of replication, a T7 phage promoter followed by a multiple cloning site and a kanamycin resistance selection marker.
- 20 2. The design of a phasmid as described in Fig. 4 containing features as in paragraph 1 or part thereof.
3. A phasmid to which a gene or a gene segment can be introduced at the multiple cloning site described therein.
4. A phasmid which can replicate in any bacterial host.
- 25 5. A phasmid which can produce single stranded DNA of the gene inserted at the multiple cloning site in any host cell upon superinfection by a helper phage.
6. A phasmid wherein a synthetic oligonucleotide containing a mismatch flanked by complementary sequences to the gene
- 30 inserted at the multiple cloning site, which produces a variant ss DNA mutagenized at a preselected spot.

7. A phasmid capable of producing ss DNA under appropriate culture conditions which can be labelled with radioactive elements such as ^{32}P or other chemical markers suitable for determining the nucleotide sequence of the ss DNA.
- 5 8. A phasmid which upon insertion into a suitable host cell, can be selected by growing the host cell in kanamycin containing medium.
9. A phasmid which contains a bacteriophage T7 promoter for transcription.
- 10 10. Any bacterial cell transformed by the phasmid which produces a protein or a peptide encoded by the gene inserted at the multiple cloning site.
11. Any bacterial cell transformed by the phasmid which produces a mutagenized protein or peptide encoded by the mutagenized ss DNA.

15

MATERIALS AND METHODS

The bacterial strains used were:

- 20 *E.coli* HB101 (F^- , hsd S20(rB^- ,mB $^-$), supE44, recA13, ara14, proA2, rpsL20(Str), xyl-1,mlt-1,);
- E.coli* JM101 (thi, (lac-proAB), [F' , traD36, proAB, lacIqZ M15]);
- E.coli* CJ236 (dut1, ung1, thi-1, relA1/pCJ105 (cam r F'));
- E.coli* BL21(DE3) (hsdS, gal (clts857 ind1 Sam7 nin5 lacUV5-T7gene1).

25

Helper phage R408.

Plasmids pET7 (a kind gift from Dr. Studier) and pGEM7Zi(+) from Promega.

30

The restriction enzymes *EcoRI*, *BamHI*, *HindIII*, *PstI*, *Sau3AI* were from Boehringer Mannheim. T4 DNA ligase, T4 DNA polymerase, Klenow fragment were from Boehringer Mannheim.

- 5 All DNA manipulations were done by essentially following protocols described in "Molecular cloning - A Laboratory Manual", Maniatis *et al.* (eds) CSH Publ., 1989.

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20
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25
11. Dwarakanath *et al.*, 1989, Gene 81, p. 219

BRIEF DESCRIPTION OF DRAWINGS

FIGURE 1

E.coli pET7 plasmid

5

FIGURE 2

E.coli pARC032 plasmid

FIGURE 3

10 *E.coli* pARC035 plasmid

FIGURE 4

E.coli pARC036 plasmid

15 FIGURE 5

Construction of pARC032 and pARC035

FIGURE 6

Construction of pARC036

20

FIGURE 7

Cloning of *E.coli st* gene in pARC036

FIGURE 8

25 *E.coli* pARC044 plasmid

FIGURE 9

Strategy for site directed mutagenesis using ss phasmid DNA as a template

CLAIMS

1. Vector phasmids pARC032, pARC035 constructed from a starting
vector incorporating
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replication origin and a morphogenetic signal, or an active mutant
thereof, of a filamentous bacteriophage of *E.coli* bacteria having F
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2. Vector phasmids pARC032, pARC035 which contain the replication
origin and a morphogenetic signal, or an active mutant thereof, of
the filamentous bacteriophage Ff (M13, fd and f1) or a mutant of
said bacteriophage.
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resistance phenotype has been replaced by kanamycin resistance
phenotype as exemplified by the construction of pARC036 from
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- 10 10. Vector phasmids pARC032 and pARC035 capable of producing ss DNA under standard culture conditions which can be labelled with radioactive elements such as ^{32}P or other chemical markers suitable for determining the nucleotide sequence of the ss DNA.
- 15 11. Vector phasmids pARC032 and pARC035 as in Claim 1 which upon insertion in a suitable host cell, can be selected by growing the host cell in kanamycin containing medium.
- 20 12. Vector phasmids pARC032 and pARC035 wherein the new restriction sites could be introduced into the multiple cloning site by standard method of *in vitro* site directed mutagenesis.
- 25 13. Vector phasmids pARC032 and pARC035 which contains a bacteriophage T7 promoter for transcription and which is transcribed *in vivo* or *in vitro* by a T7 RNA polymerase.
14. A bacterial cell, transformed by the vector phasmids pARC032 or pARC035, which produces a protein or a peptide encoded by a gene inserted at the multiple cloning site.
- 30 15. A bacterial cell, transformed by the vector phasmids pARC032 or pARC035, which produces a mutagenized protein or peptide encoded by the mutagenized ss DNA.

16. A process for the production of a protein or a peptide, encoded by the gene inserted at the multiple cloning site, by using a bacterial cell transformed by the vector phasmids pARC032 or pARC035.
- 5 17. A process for the production of a mutagenized protein or peptide, encoded by the mutagenized ss DNA, by using a bacterial cell transformed by the vector phasmids pARC032 or pARC035.

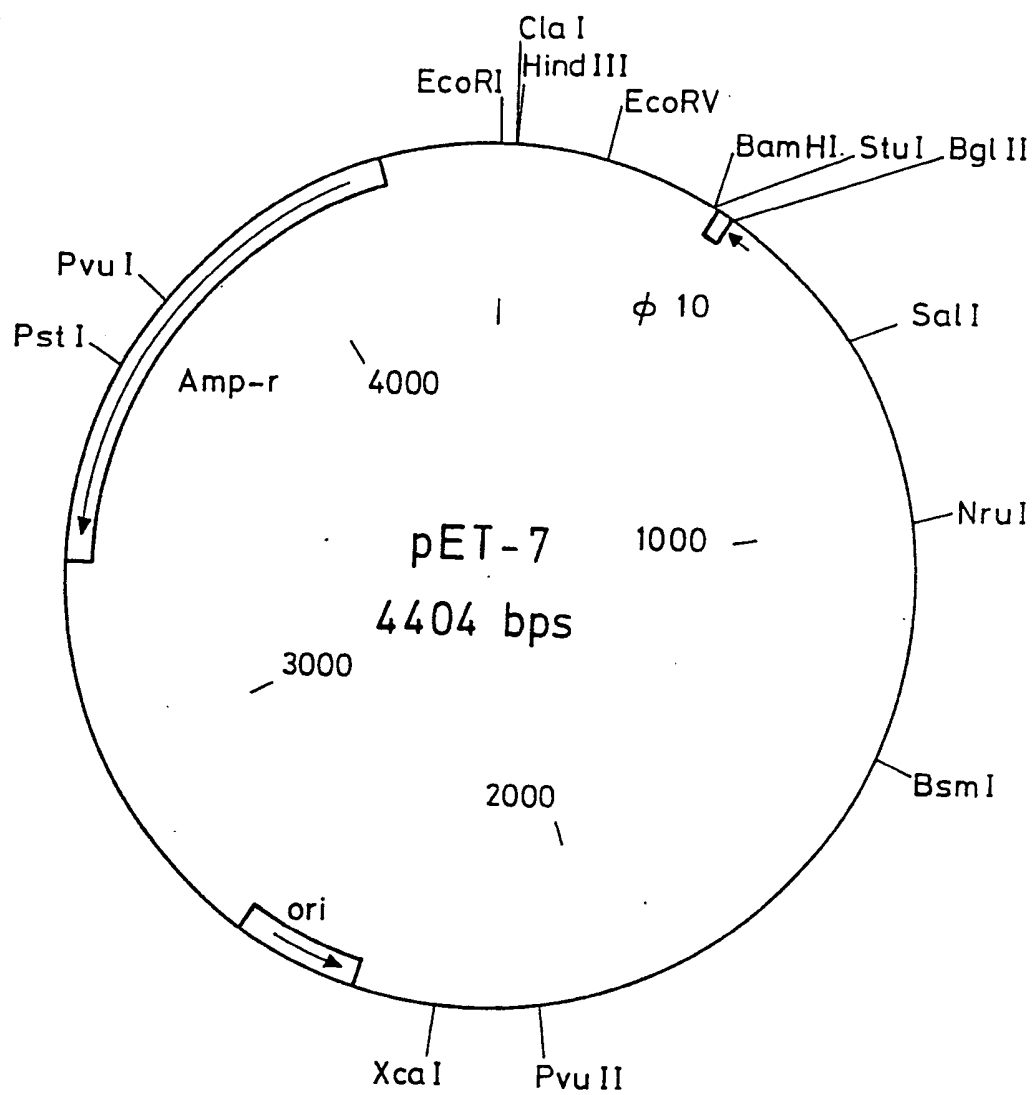


Fig. 1

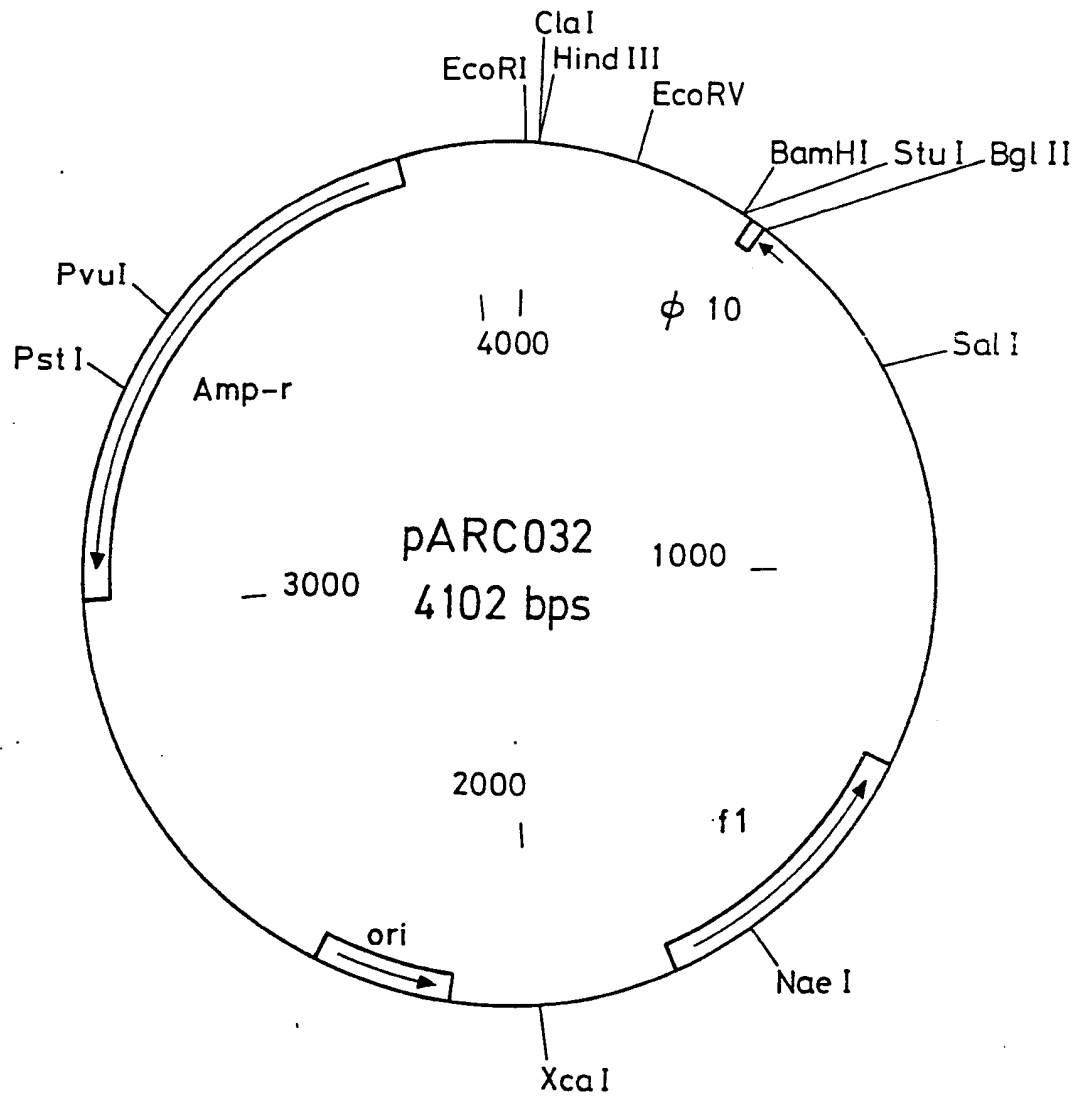


Fig. 2

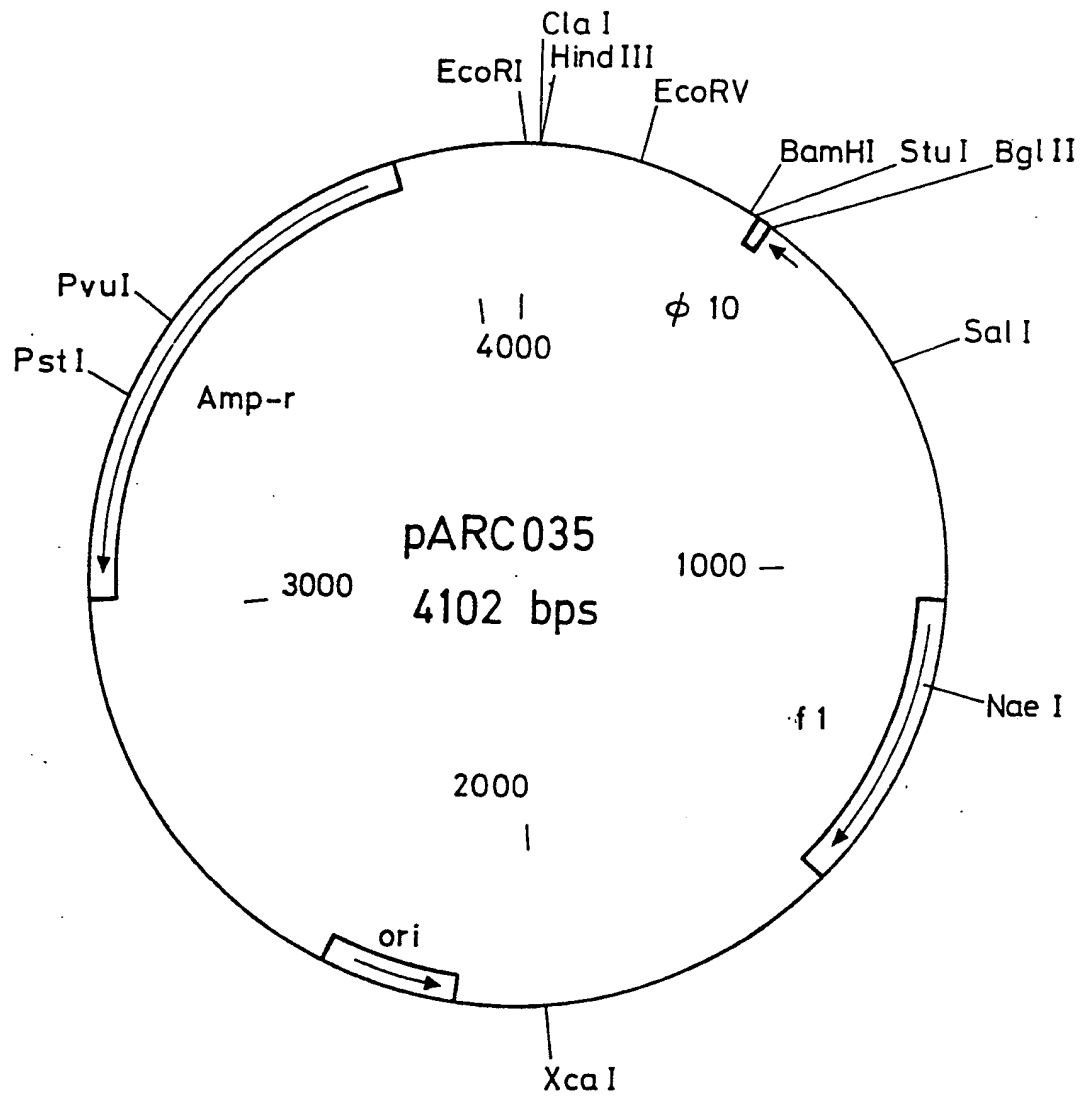


Fig. 3

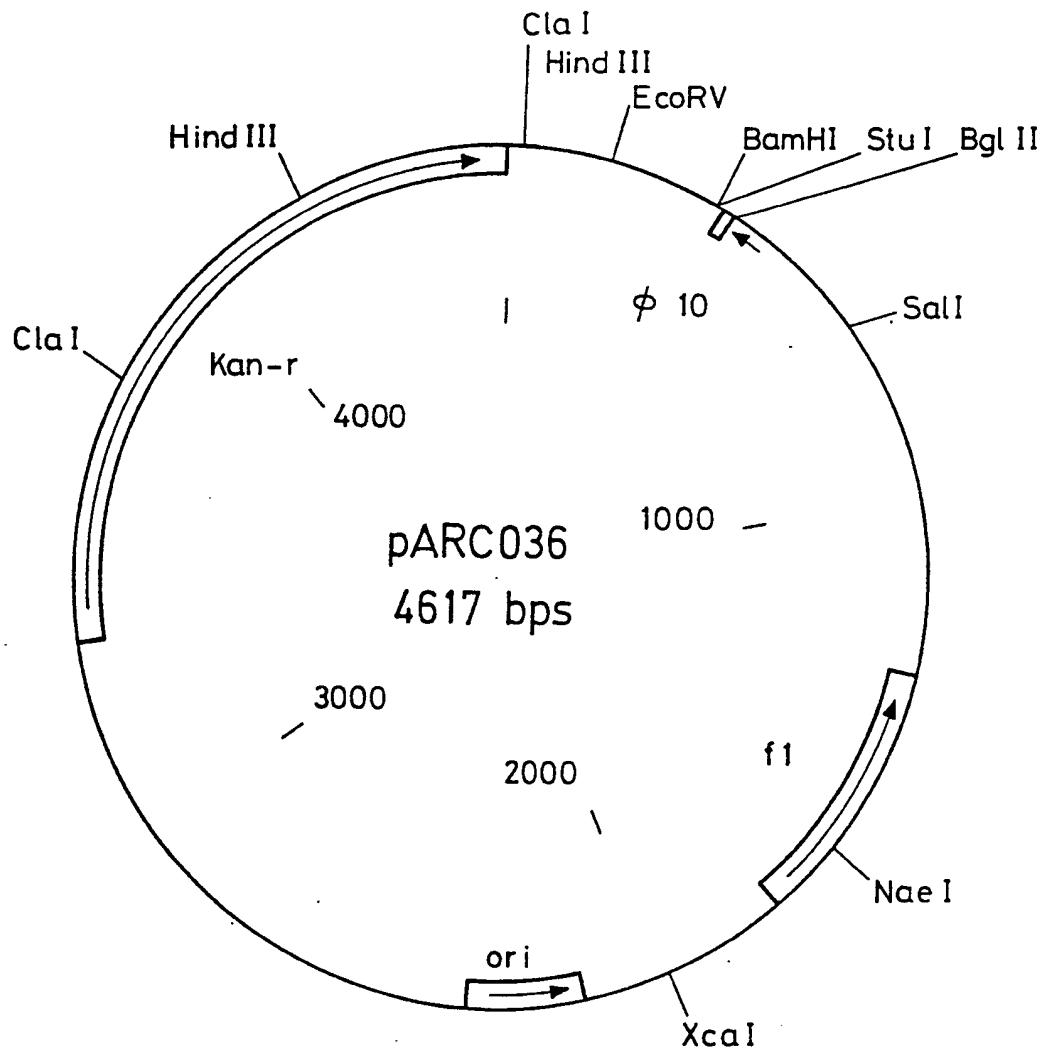
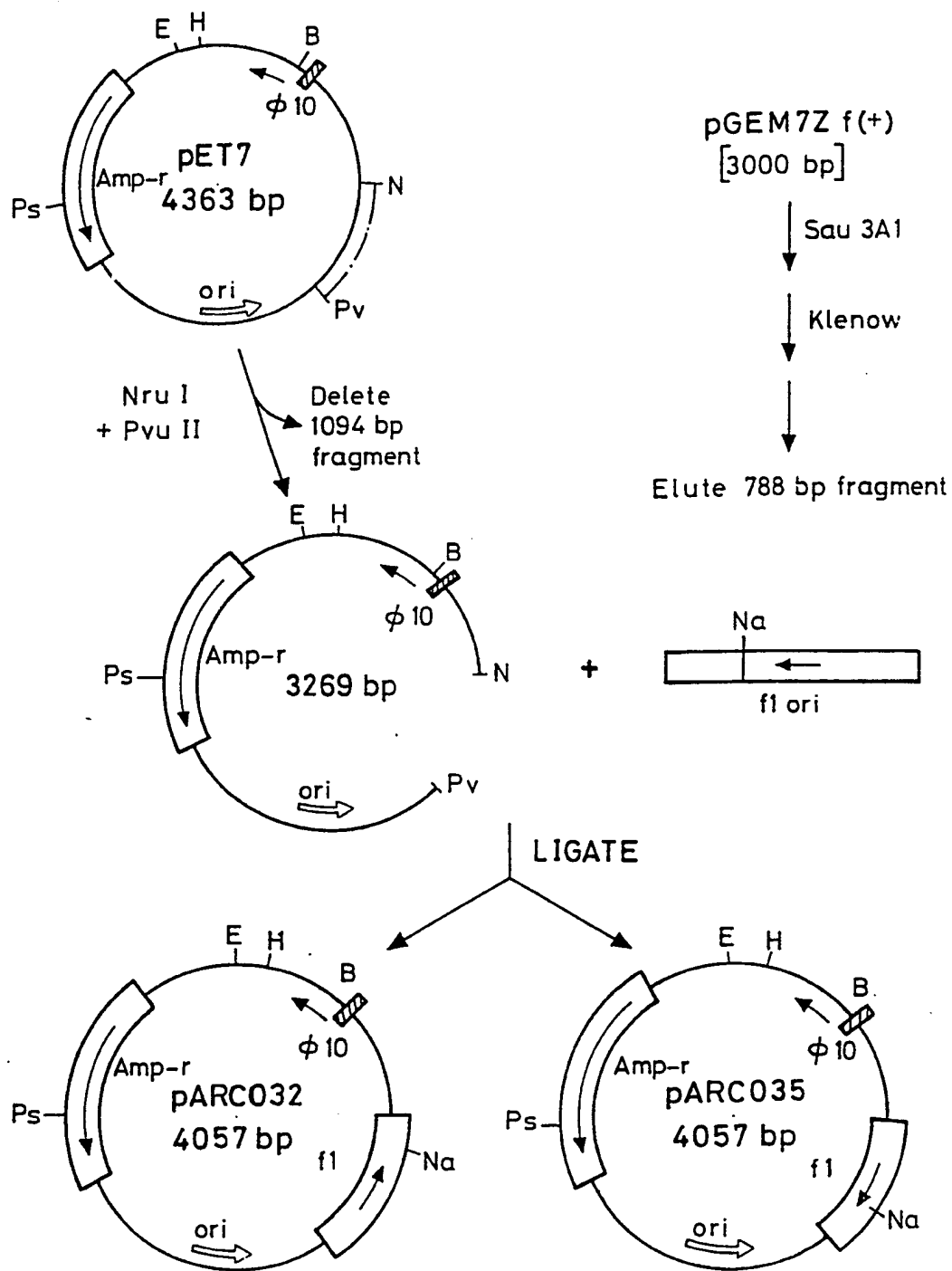


Fig. 4

Cloning of f1 origin of replication in pET7



E = EcoRI	Pv = Pvu II
Ps = Pst I	Na = Nae I
B = BamHI	H = Hind III
N = Nru I	

Cloning of Kan-r gene cartridge in pARC032

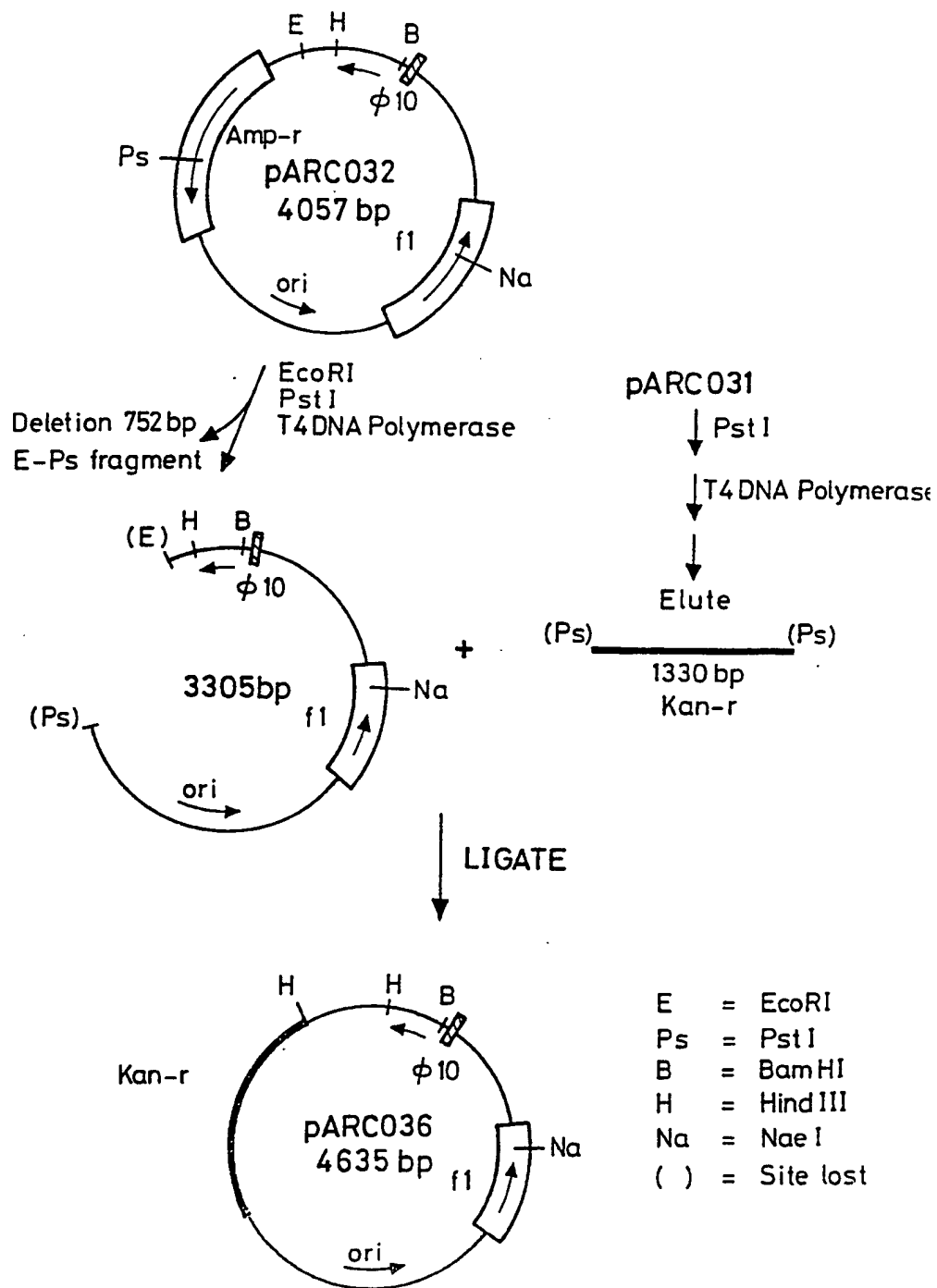


Fig. 6

Cloning of st gene in phasmid vector pARC 036

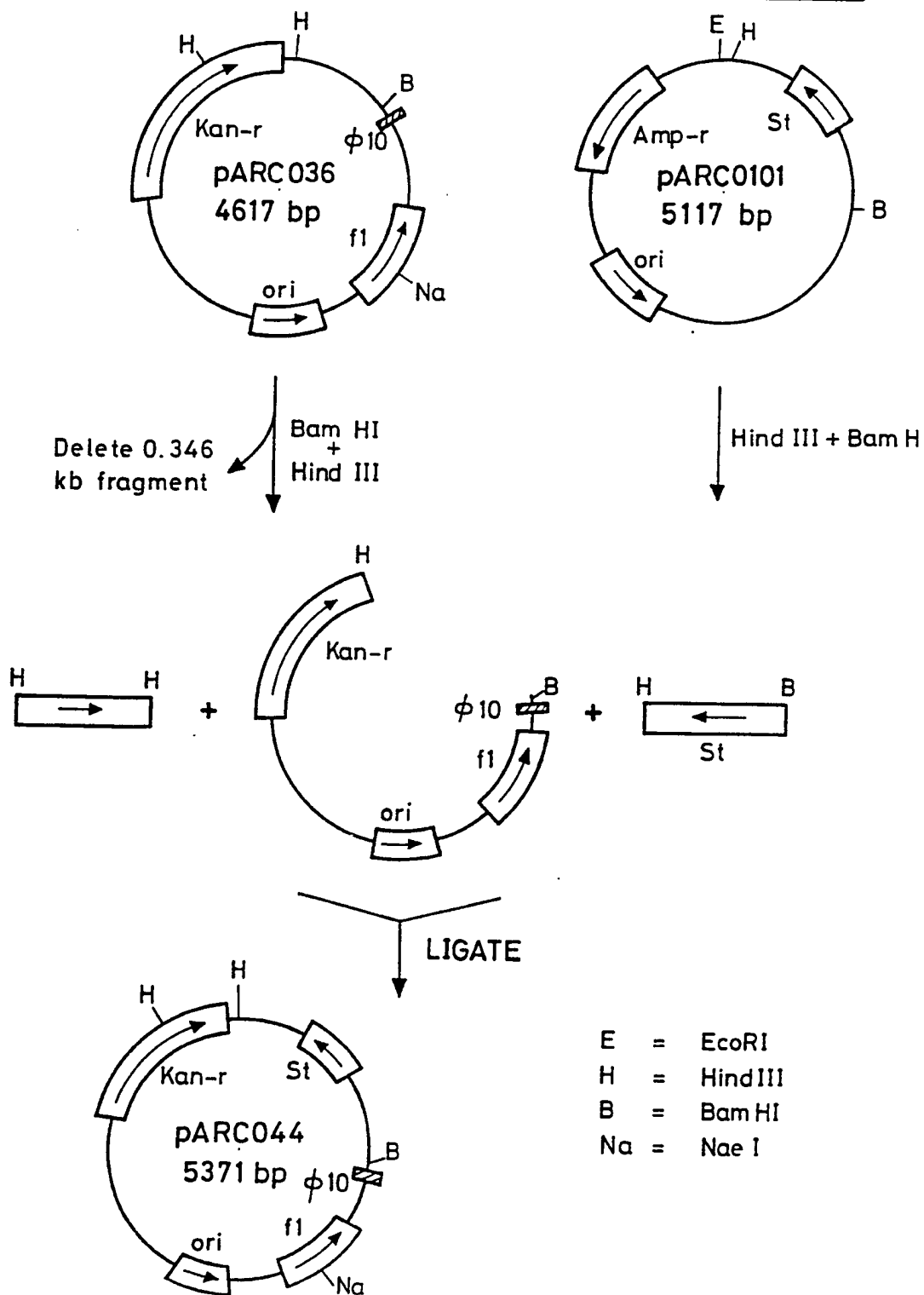


Fig. 7

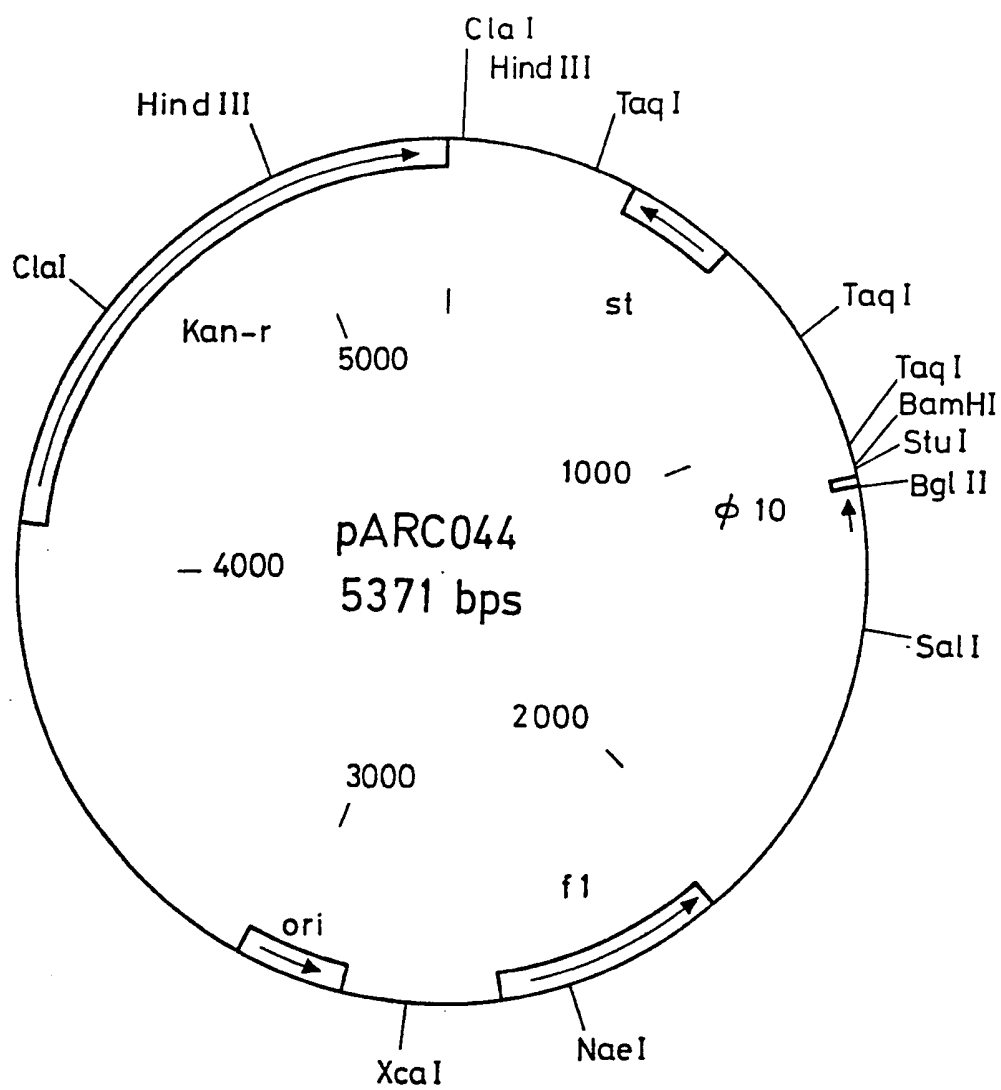


Fig. 8

Strategy to replace Tyr-72 with Phe-72 in *E. coli* *st* gene

Transform *E. coli* CJ 236 with pARC 044

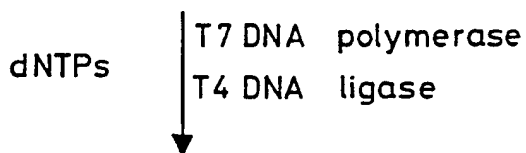
Infect with helper phage R 408



ss phasmid → pARC044

wt. *st* gene :- 5'ATA TTA TTA ATA GCA CCC CG - 3'

mutant primer :- 5'ATA TTA TTA A[A]A GCA CCC GG - 3'



Transform DNA into *E. coli* HB 101 *rec*⁻ competent cells



Check mutant phasmid by DNA sequencing

Fig. 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 92/00793

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C12N 15/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Gene, Volume 90, 1990, Takayasu Date et al, "Construction of Escherichia coli vectors for expression and mutagenesis: synthesis of human c-Myc protein that is initiated at a non-AUG codon en exon 1", page 141 - page 144, see especillay the abstract and fig. 1 --	1-17
X	Gene, Volume 105, 1991, Barry March Forman et al, "pExpress: A family of expression vectors containing a single transcription unit active in prokaryotes, eukaryotes and in vitro", page 9 - page 15, see especially the abstract and fig. 2 --	1-17

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE 92/00793

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, file 154, Medline, accession no. 06882389, Medline accession no. 89184389, Mead D.A. et al: "Single-stranded DNA "blue" T7 promoter plasmids: a versatile tandem promoter system for cloning and protein engineering", --	1-17
X	DE, A1, 3929822 (MAX-PLANCK-GESELLSCHAFT ZUR FÖRDERUNG DER WISSENSCHAFTEN EV), 14 March 1991 (14.03.91), see the claims --	1-17
X	EP, A2, 0354727 (ELI LILLY AND COMPANY), 14 February 1990 (14.02.90), see fig. 2 --	1-17
A	EP, A2, 0356130 (EASTMAN KODAK COMPANY), 28 February 1990 (28.02.90), see the whole document -- -----	1-17

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Information on patent family members

29/01/93

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DE-A1- 3929822	14/03/91	NONE	
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